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Systematics of the *Etheostoma cinereum* (Teleostei: Percidae) species complex (subgenus *Allohistium*)

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Abstract

We examined geographic variation within the Ashy Darter, *Etheostoma cinereum,* of the mitochondrially enconded cytochrome *b* gene (cyt *b*) and nuclear recombination activation gene 1 (RAG1) as well as pigmentation, 6 meristic variables, and 20 morphometric variables for patterns indicative of speciation within the complex. Four geographically disjunct entities were identified by at least one of the datasets corresponding to the Cumberland, Duck, Elk, and upper Tennessee river systems. Monophyly of cyt *b* and RAG1 sequences, modal meristic differences, moderate morphometric divergence, and unique pigmentation in specimens from the Cumberland River suggest this entity represents an evolutionary species under many different species concepts and is described herein as *Etheostoma maydeni*. Other populations exhibit varying degrees of divergence in the different datasets and have conflicting relationships in phylogenetic analyses using cyt *b* and RAG1 sequences, leaving the evolutionary history and taxonomic status of the Duck, Elk and upper Tennessee populations unclear.

Key words: *Etheostoma maydeni,* Cumberland River, endemic, darter

Introduction

The Ashy Darter, *Etheostoma cinereum* Storer was described from specimens collected near Florence, Alabama, in the Tennessee River system (Storer 1845). Little formal investigation into the distribution of and variation within this species occurred prior to Shepard & Burr (1984) who found the species extirpated from much of its historic range and morphological variation suggesting the species was composed of "…three somewhat distinct populations: Cumberland drainage, Duck drainage, and upper Tennessee drainage." Declining populations (Powers & Mayden 2002) in need of active management triggered an investigation of genetic diversity within and among populations for cytochrome b (cyt *b*) that revealed genetically divergent management units (MU) restricted to the three major river systems with extant populations of *E. cinereum*: Cumberland, Duck, and Upper Tennessee rivers (Powers *et al.* 2004). The genetic divergence of these MUs coinciding with morphological divergence identified by Shepard & Burr (1984) suggests cryptic biodiversity within *E. cinereum.* The lack of type specimens (Collette & Knapp 1966) and apparent extirpation from the Tennessee River proper and any tributary within 300 rkm of the type locality of Florence, AL, left resolving the systematics of these MUs problematic. Recent rediscovery of *E. cinereum* in the Elk River provided not only an opportunity to examine this population for morphological and genetic variation, but also provided a surrogate to topotypic *E. cinereum* as the Elk confluences with the Tennessee River less than 50 rkm from Florence with no clear biogeographic barriers between them, thus making systematic revision less uncertain. The objectives of this study were to compare meristic, morphometric, pigment, and genetic variation within and among extant populations of the *E. cinereum* complex (Fig. 1) in order to test the hypothesis that *E. cinereum* represents a single evolutionary species.

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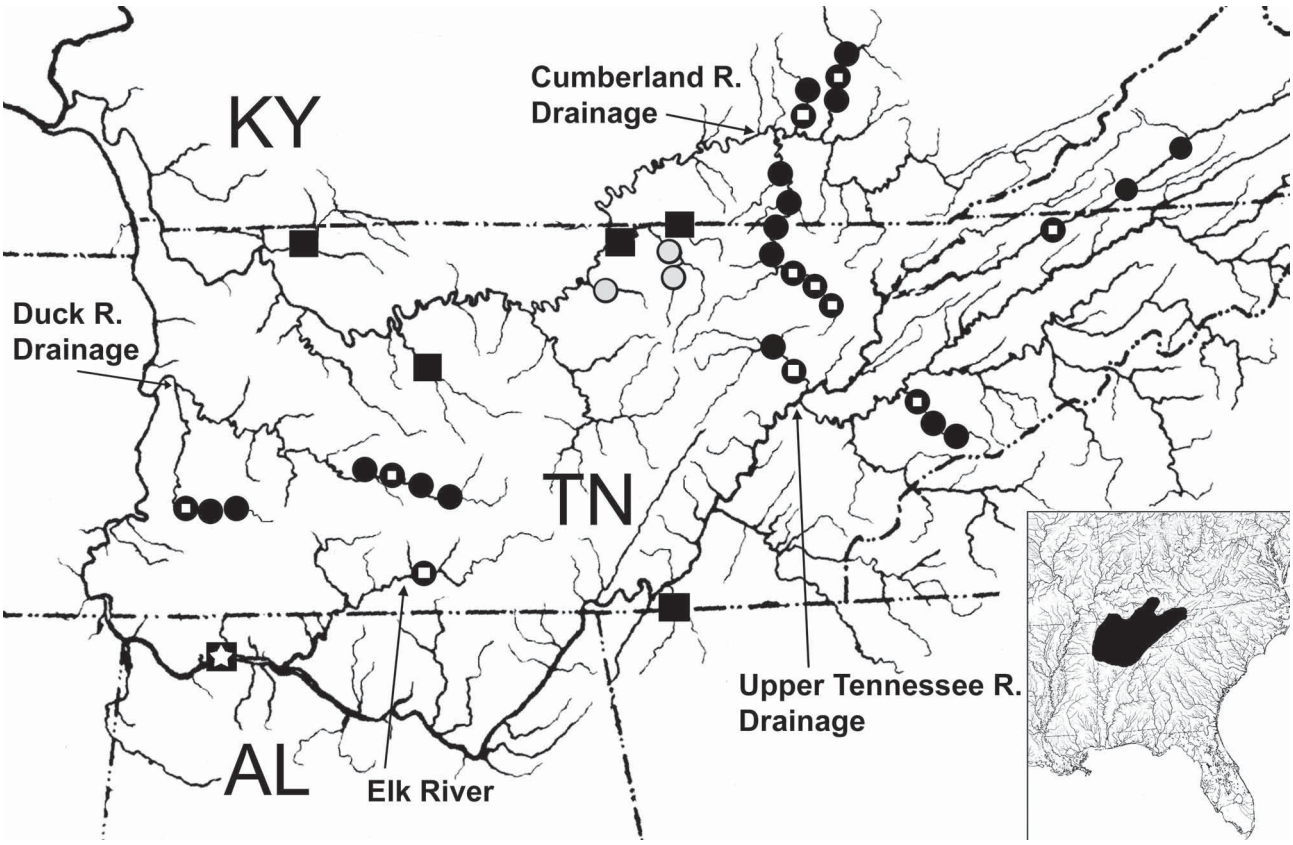

FIGURE 1. Range of *Etheostoma cinereum* species complex. Squares indicate historic localities where populations are most likely extirpated . Square with white star is type locality for *E. cinereum*, grey circles indicate historic localities that appear to have appropriate habitat but no specimens have been observed since early 1970s, black dots indicate recent collections, black dots containing white squares indicate localities from which specimens were examined. Extant Cumberland populations are described herein as *E. maydeni.*

Methods

Morphological analyses

Variation of the *Etheostoma cinereum* species complex was evaluated using meristic characters following Hubbs & Lagler (2004) except for transverse scales which were counted from the origin of the anal fin anteriodorsally to the base of the spinous dorsal fin. Six meristic characters regularly variable among closely related species of darters and found to be variable by Shepard & Burr (1984) were examined: dorsal fin spines, dorsal fin rays, anal fin rays, lateral-line scales, transverse scales, and caudal peduncle scales. All meristic data were collected from 117 specimens. Frequency distribution tables were generated for meristic variables and examined for modal differences among samples from the Cumberland, Duck, Elk, and Upper Tennessee (Table 1). An analysis of variance (ANOVA) with interactions was performed for meristic counts among the above drainages (alpha = 0.05). Meristic data were analyzed in Data Desk 6.0 (Data Description, Inc., Ithaca, NY). In "Description," the number of individuals with a given count is in parentheses following the count.

Morphometric variation was assessed by standard and truss measurements generally following Hubbs & Lagler (2004) and Humphries *et al.* (1981), respectively. Twenty standard and truss measurements were recorded from 110 specimens greater than 50 mm SL using electronic calipers under 6x magnification and input directly into an Excel spreadsheet (Microsoft Corp., Redmond, WA). Measurements examined (with corresponding landmarks as noted in Fig. 2 to ensure homology among measurements) were standard length (1–23), diameter of orbit (2–3), head length (1–4), predorsal length (1–5), tip of snout to origin of pelvic fin (1–6), interorbital width (2–2), origin of spinous-dorsal fin to origin of pelvic fin (5–6), pectoral fin length (7–8), width at pectoral fins (7–7), length of spinous-dorsal fin base (5–11), length of sixth dorsal spine (9–10), pelvic fin length (6–12), origin of soft-dorsal fin to origin of anal fin (13–14), origin of soft-dorsal fin to dorsal origin of caudal fin (13–21), length of soft-dorsal fin

base (13–19), origin to most distal point of soft dorsal fin (13–20), origin of anal fin to ventral insertion of caudal fin (14–22), length of eighth dorsal ray (15–16), length of fourth anal ray (17–18) and depth of caudal peduncle (21–22). Analysis of morphometric data followed Armbruster & Page (1996) as raw mensural data were natural log-transformed and a principal components (PC) analysis using the covariance option was performed in Data Desk 6.0 (Data Description, Inc., Ithaca, NY). Size of specimens was accounted for by PC 1; thus, scatterplots of PC 2 and PC 3 were examined for morphological divergence and geographic trends. Due to sexual dimorphism in *E. cinereum* (Shepard & Burr 1984), morphometric data for males and females were analyzed separately.

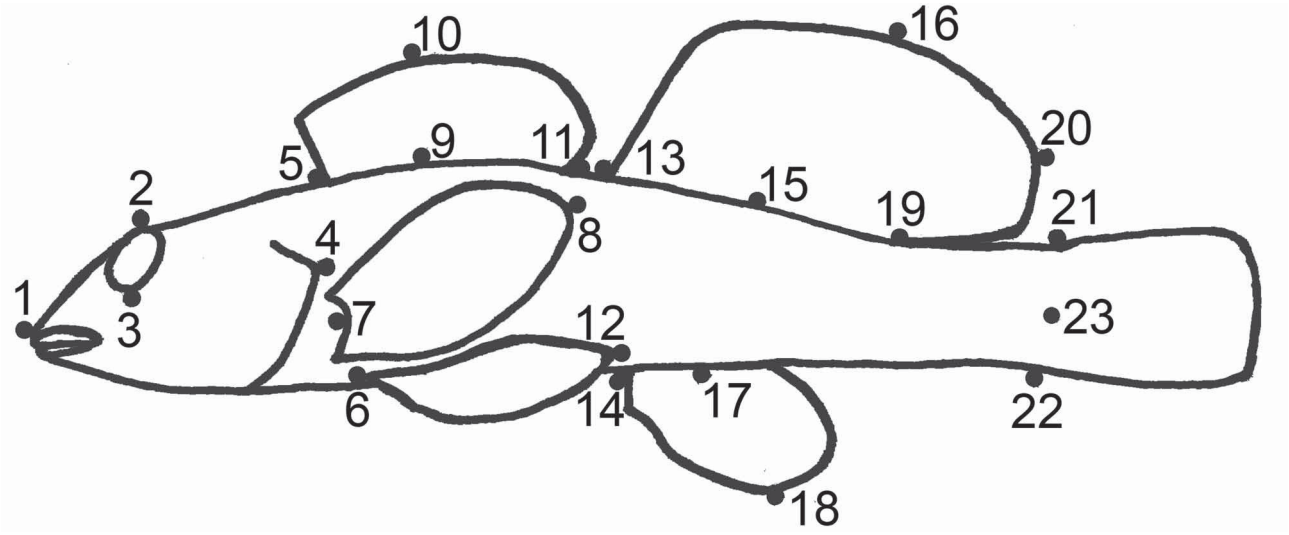

FIGURE 2. Landmarks used for morphometric evaluation of *Etheostoma cinereum* species complex.

Details of pigmentation were obtained by qualitative examination of live specimens, color photographs of nuptial specimens, and recently preserved specimens from multiple localities within the Duck, Cumberland, and Tennessee river systems. All descriptions of pigmentation are of live nuptial males unless otherwise noted.

In the list of type specimens, the number of types in a lot is listed, followed by a comma, and SL of specimens. Specimens examined for this study are listed in Materials Examined in which each catalog number is followed by a comma and number of specimens from which data were taken. Institutional abbreviations follow Sabaj Pérez (2010).

Molecular analyses

Complete and partial mitochondrial cyt *b* sequence (1140 base pairs) was obtained from all extant populations the *E. cinereum* (n = 41): three localities in the Cumberland River drainage; Rockcastle River, Kentucky (n = 2), New River, Tennessee (n = 7), Buck Creek, Kentucky (n = 1); two localities in the Duck River drainage in Tennessee, Duck River (n = 11) and Buffalo River (n = 1); three localities in the upper Tennessee River drainage in Tennessee, Little River (n = 2), Clinch River (n = 8), and Emory River (n = 1); one locality in the Elk River (n = 8). Sequence of the nuclear recombination activation gene-1 (RAG1) was obtained from a subsample of specimens (n = 8) representing the range of the species and included specimens from the Cumberland River drainage, Rockcastle River, Kentucky (n = 1), New River, Tennessee (n = 1); the Duck River (n = 2); the upper Tennessee River drainage, Clinch River (n = 1), Emory River (n = 1); and the Elk River (n = 2). Museum and GenBank accession numbers are provided in Materials Examined. Whole genomic DNA was extracted from frozen or ethanol preserved specimens using standard phenol-chloroform methods. The cyt *b* and RAG1 genes were amplified with 30 cycles of PCR using protocols and primers designed by Song *et al.* (1998) and Lopez *et al.* (2004), respectively. Denaturation, annealing, and extension temperatures and times were: 95o C, 40 sec; 55o C, 60 sec; and 72o C, 90 sec, respectively. Amplified PCR products were purified by centrifugal filtration using the GenElute PCR Clean-Up Kit (Sigma-Aldrich Inc., St. Louis, MO) following manufacturers' directions. Sequencing was conducted by Virginia Bioinformatics Institute, Blacksburg, VA. Sequences were aligned by eye, and ambiguous bases were labeled as "n" using BioEdit (Hall 1999). No gaps were needed for alignment.

Monophyly of cyt *b* haplotypes of the *E. cinereum* was tested by including sequence data from *Ammocrypta beanii* (AF386535), *Percina macrocephala* (AF386592), *P. peltata* (AF386596), *P. burtoni* (AF386554), *P. copelandi* (AY374283), *P. tanasi* (AF386578), *P. maculata* (AF386557), *P. stictogaster* (AF045355), *P. squamata* (AF386564), *P. aurolineata* (AF386575), *P. sciera* (AF386574), *P. aurantiaca* (AF386580), *P. evides* (AF386580), *Sander canadense* (AF386603), *Etheostoma simoterum* (GenBank DQ089050), *E. sagitta* (AF045343), *E. stigmaeum* (AY374276), *E. microperca* (FJ381003), *E. blennioides* (AF288426), *E. nigrum* (GQ183677), *E. flabellare* (AF045342), and *E. rufilineatum* (GU015278) as outgroup taxa representing a non-darter percid and most subgenera of darters. Use of a large number of outgroups for the cyt *b* analysis is due to previous studies identifying introgression of mtDNA in darters (Ray *et. al.* 2008; Bossu & Near 2009). Outgroups for the RAG1 analysis were *Sander vitreus* (FJ381300), *Etheostoma rupestre* (JF742879), and *E. simoterum* (JF497517). For each gene, variation within and among drainages was examined by calculating pairwise distances using MEGA (Tamura *et al.* 2007). Phylogenetic hypotheses were generated with maximum parsimony in NONA (version 2, Goloboff, P., NONA {NO NAME}, Tucumán, Argentina, 1999, unpubl.). Heuristic searches were conducted using all characters equally weighted and 50 replications of the random addition sequence option. Branches with lengths of zero were collapsed. Support for hypotheses was evaluated by performing 1000 bootstrap replicates (Felsenstein 1985) in NONA and decay analyses (Bremer 1994) with Sepal (version 1.4, B. A. Salisbury, SEPAL: strongest evidence and parsimony analyzer, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, 2000, unpubl.). Bayesian analyses were conducted using MrBayes (version 3.1.2) following the "Quick Start" settings outlined in the manual (Ronquist & Huelsenbeck 2003).

Results

Frequency distributions with means for lateral-line scales, dorsal spines, dorsal rays, anal rays, transverse scales, and caudal-peduncle scales are presented in Table 1. Modal counts of specimens from each river system are in bold font. The null hypothesis was rejected for each of these variables in an ANOVA with F and p-values listed, respectively: 9.2, <0.0001; 13.9, <0.0001; 9.7, <0.0001; 3.1, 0.03; 5.4, 0.002; 5.8, 0.0001.

TABLE 1. Frequency distribution of lateral-line scales (LLS), dorsal spines (DS), dorsal rays, (DR), anal rays (AR), transverse scales (TS), and caudal peduncle scales (CPS) for *Etheostoma cinereum* complex specimens (n = 117) from the Cumberland River system, herein described as *E. maydeni,* Duck River system, Elk River system, and Upper Tennessee River system (U Tenn).

LLS	50	52	53	54	55	56	57	58	59	60	61	62	63	64	mean
E. maydeni	0	2	4	3	2	4	5	**6**	4	4	3	2	1	0	57.3
Duck	0	0	0	3	0	4	4	5	**10**	4	3	5	3	0	58.9
Elk	0	0	0	0	0	0	2	2	0	1	**4**	0	2	0	60.0
U Tenn	2	0	4	3	**6**	1	2	2	2	1	0	1	0	1	55.8

DS	10	11	12	13	mean	DR	11	12	13	14	mean
E. maydeni	0	**29**	11	0	11.3		0	**30**	10	0	12.3
Duck	0	9	**27**	5	11.9		0	13	**26**	2	12.7
Elk	0	1	**9**	1	12.0		1	**5**	**5**	0	12.4
U Tenn	1	2	**17**	5	12.0		0	6	**15**	4	12.9

AR	7	8	9	mean	TS	17	18	19	20	21	22	23	mean
E. maydeni	3	**27**	10	8.2		0	3	4	**13**	9	6	5	20.7
Duck	0	**27**	14	8.3		0	0	9	**12**	**12**	7	1	20.5
Elk	0	**11**	0	8.0		0	1	3	**4**	2	1	0	19.9
U Tenn	3	**19**	3	8.0		2	**6**	**6**	**6**	1	4	0	19.4

CPS	21	22	23	24	25	26	27	mean
E. maydeni	1	7	**15**	7	8	2	0	23.5
Duck	0	2	8	8	**10**	**10**	3	24.7
Elk	0	0	3	2	**5**	0	1	24.5
U Tenn	0	2	5	**8**	4	6	0	24.3

In the principal components analysis of morphometric data, for males, PC 2 loaded heavily for length of sixth dorsal spine (-0.30), origin of anal fin to ventral insertion of caudal fin (0.41), and length of eighth dorsal ray (-0.57), while PC 3 loaded heavily for width at pectoral fins (0.40), length of sixth dorsal spine (0.39), origin of soft-dorsal fin to dorsal origin of caudal fin (-0.33), origin of anal fin to ventral insertion of caudal fin (-0.42), and length of eighth dorsal ray (-0.39). For females, PC 2 loaded heavily for origin of spinous-dorsal fin to origin of pelvic fin (-0.32), pectoral fin length (-0.38), length of sixth dorsal spine (-0.33), origin of soft-dorsal fin to dorsal origin of caudal fin (0.35), and origin of anal fin to ventral insertion of caudal fin (0.42), while PC 3 loaded heavily for width at pectoral fins (0.87) and length of eighth dorsal ray (-0.36). Scatterplots of PC 2 and PC 3 for males (Fig. 3) have Upper Tennessee and Elk specimens forming clusters without overlapping specimens from any other drainage. Cumberland and Duck specimens have moderate to low overlap between them with divergence mostly along PC 2. Scatterplots of PC 2 and PC 3 for females (Fig. 4) have near complete overlap between Upper Tennessee and Elk specimens with both having moderate to low overlap with Duck specimens and no overlap with Cumberland specimens with divergence mostly along PC 2. Duck specimens broadly overlap with Cumberland specimens.

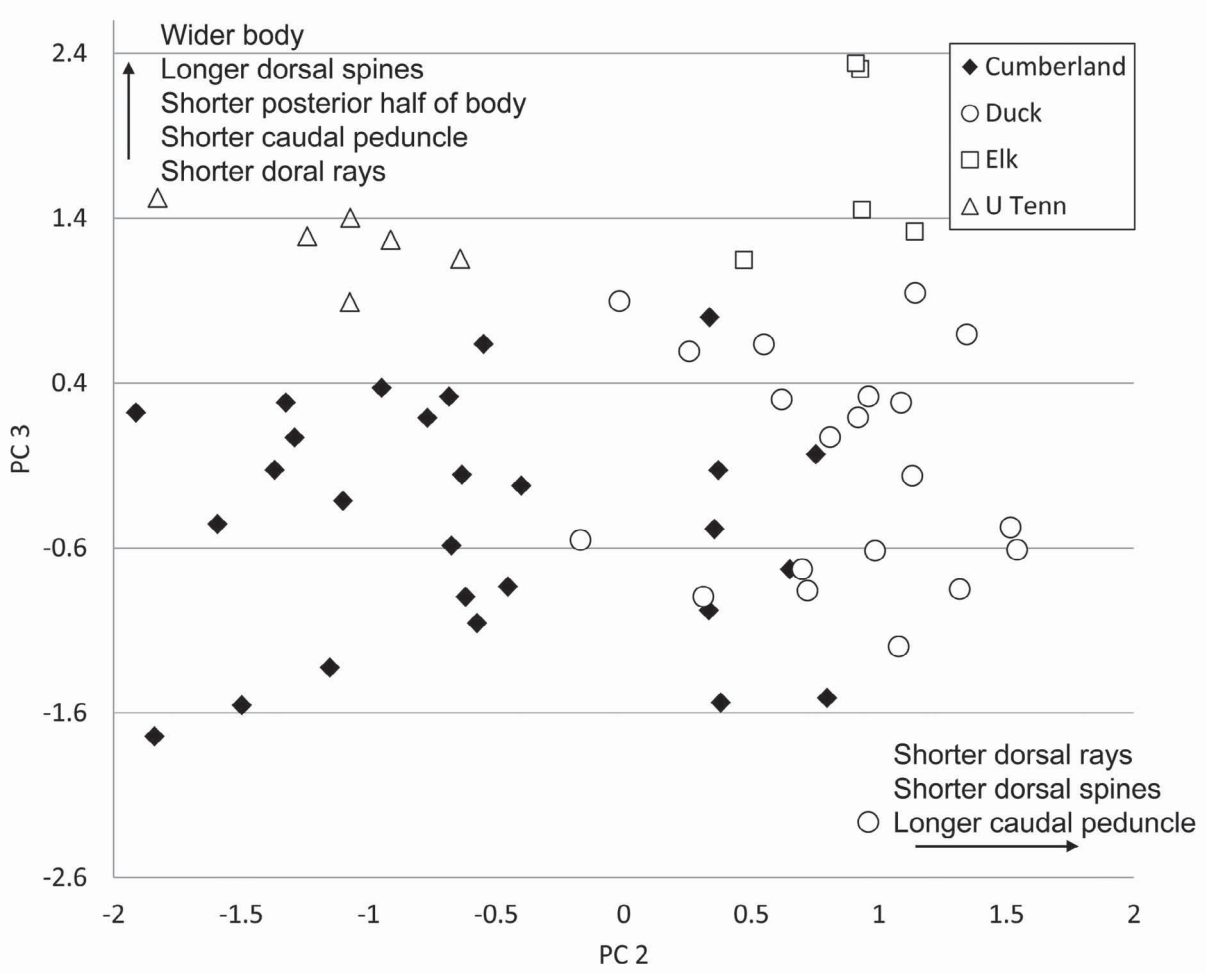

FIGURE 3. Scatterplot of second and third principal component scores of natural log-transformed morphometric characters of the *Etheostoma cinereum* species complex adult males (n = 59). Cumberland specimens are described herein as *E. maydeni.*

Variation in pigmentation of fins or the body of specimens appeared to show no geographic patterns, but pigmentation around the mouth showed strong geographic pattern (Fig. 5 A–D). All nuptial males from the Cumberland drainage had conspicuous red pigment covering most (approximately 75% or more) of the external surface of the lips. Cumberland juvenile males and females also had conspicuous red pigment on the lips during the spring breeding season, and the pigment persisted even months removed from spawning. No red pigment was observed on the lips of any specimen examined from the upper Tennessee including peak nuptial males. A rust to faint red pigment covered a small amount (approximately 20% or less) of the visible surface area of the lips in the largest nuptial males examined from the Duck and Elk rivers, but was completely absent from females and non-nuptial males.

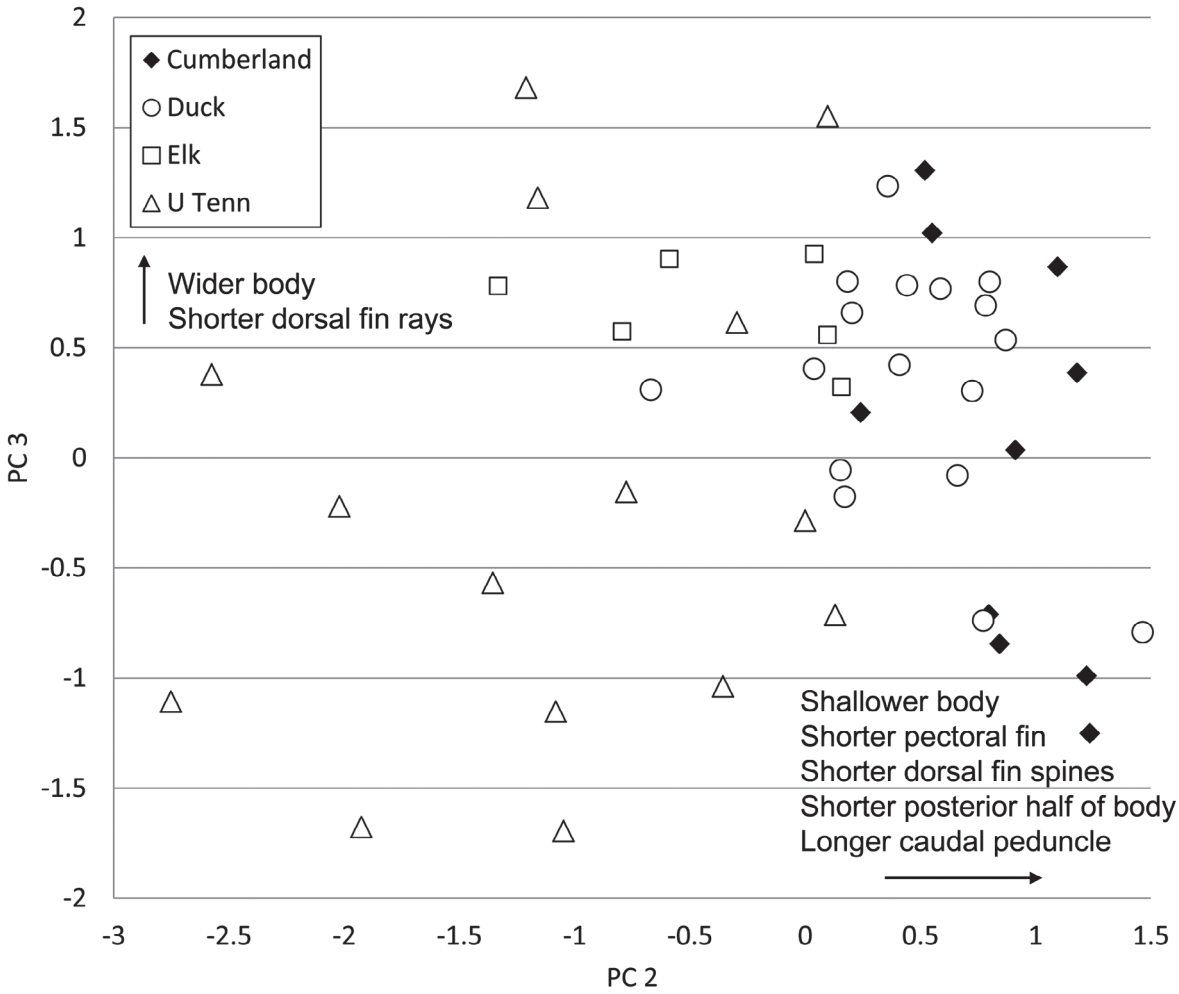

FIGURE 4. Scatterplot of second and third principal component scores of natural log-transformed morphometric characters of the *Etheostoma cinereum* species complex adult females (n = 51). Cumberland specimens are described herein as *E. maydeni.*

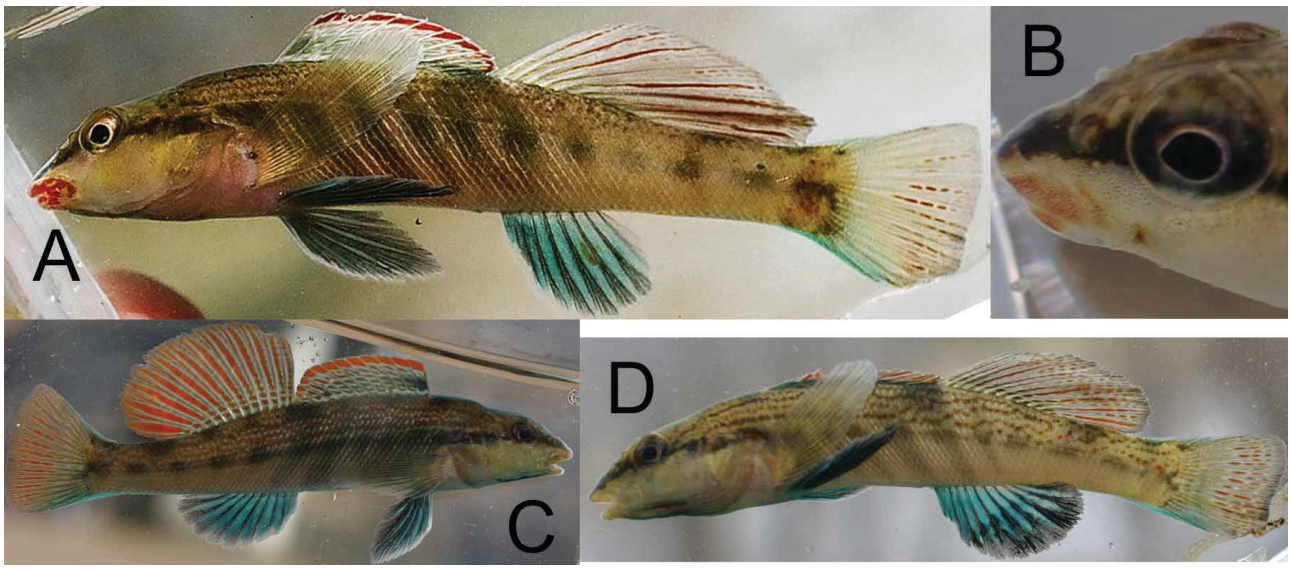

FIGURE 5. A. Nuptial male *Etheostoma maydeni* (USNM 403641, 79.7 mm SL, holotype), New River at U.S. Hwy 27 near New River, Scott County, Tennessee, 7 March 2006. B. Male *E. maydeni* (UAIC 15058.01, 42.3 mm SL) New River at U.S. Hwy 27 near New River, Scott County, Tennessee, 4 March 2005. C. Nuptial male *E. cinereum* (UAIC 15056.01, 82.6 mm SL), Duck River at Shelbyville, Bedford County, Tennessee, 3 March 2005. D. Nuptial male *E. cinereum* (UAIC 15062.01, 70.1 mm SL), Clinch River at Frost Ford, Hancock County, Tennessee, 8 March 2006.

Of the 1140 base pairs examined for cyt *b*, 377 sites were variable, 160 of which were parsimony-informative. Among the 41 *E. cinereum* individuals sequenced, 16 haplotypes were recovered. A maximum parsimony analysis identified 40 equally parsimonious trees with a length of 2150 steps (CI = 0.34, RI = 0.63). The strict consensus tree including all outgroups with clades of *E. cinereum* complex identified but collapsed for clarity is presented in Fig. 6, and the consensus tree of only *E. cinereum* complex specimens is presented in Fig. 7. The Bayesian analysis produced a similar topology with only minor differences within each major clade. All *E. cinereum* haplotypes formed a monophyletic group with 100% bootstrap support, a decay value of 48, and posterior probability >95%. Haplotypes from the Duck, Cumberland and Upper Tennessee with the latter including specimens from the Elk were resolved as clades with moderate to high bootstrap and decay support (Fig. 7). The Duck River clade was recovered as sister to a clade containing the Cumberland and Upper Tennessee clades with moderate bootstrap and no decay support. Relationships of individuals within each clade were largely unresolved or were not concordant with geographic distribution. No haplotypes were shared among major clades. Divergence of haplotypes within each clade was 0.1%. Mean divergence among each clade was 1.2%. The greatest mean divergence among clades was between the Duck and Cumberland (2.7%). Mean divergence of Upper Tennessee and Duck clades was 2.3%. The smallest mean divergence was between the Upper Tennessee and Cumberland clades (1.0%).

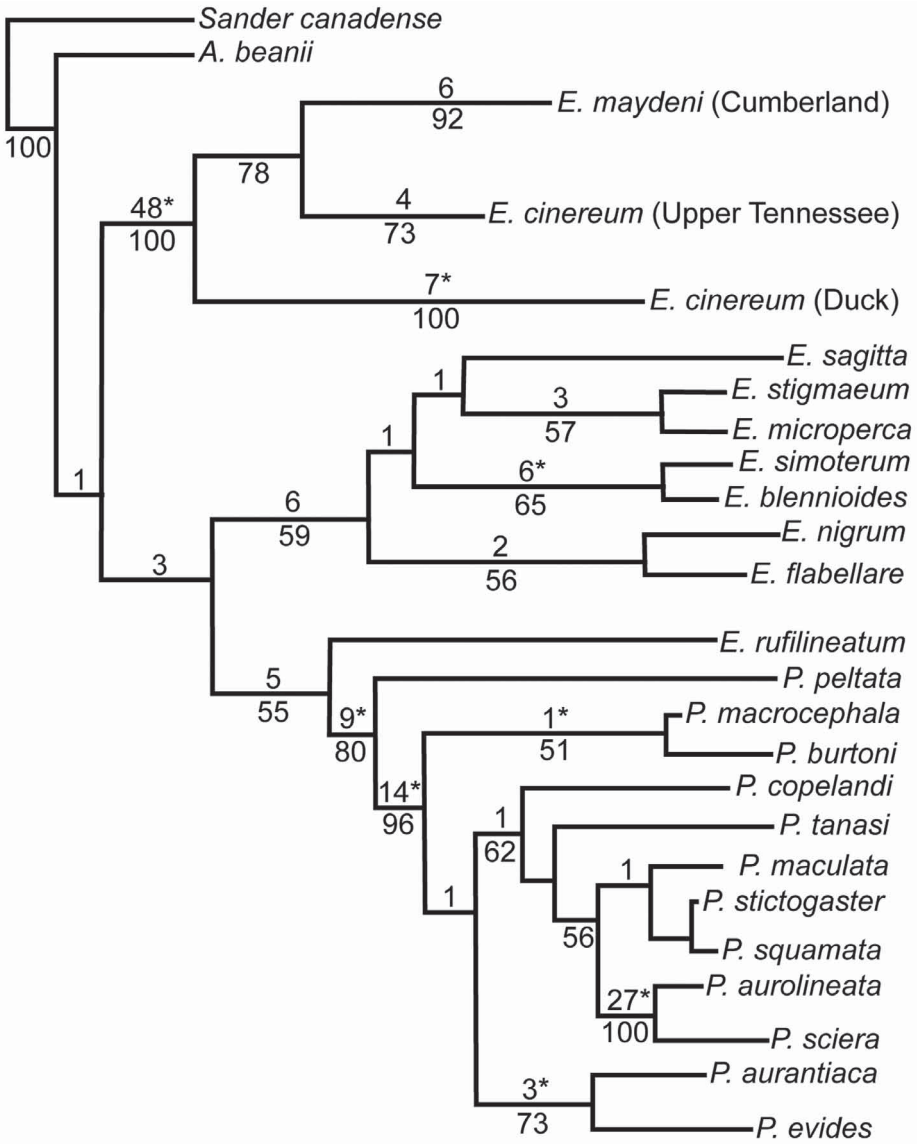

FIGURE 6. Strict consensus of 40 equally parsimonious trees with a length of 2150 steps (CI = 0.34, RI = 0.63) generated by analysis of cytochrome *b* sequence data. Bootstrap and decay support is listed above and below each internode, respectively. An asterisk indicates nodes recovered with posterior probability values >95% in a Bayesian analysis with a similar topology. Each clade of *Etheostoma cinereum* complex haplotypes is collapsed for clarity. All *E. cinereum* cyt *b* haplotypes are presented in Figure 7.

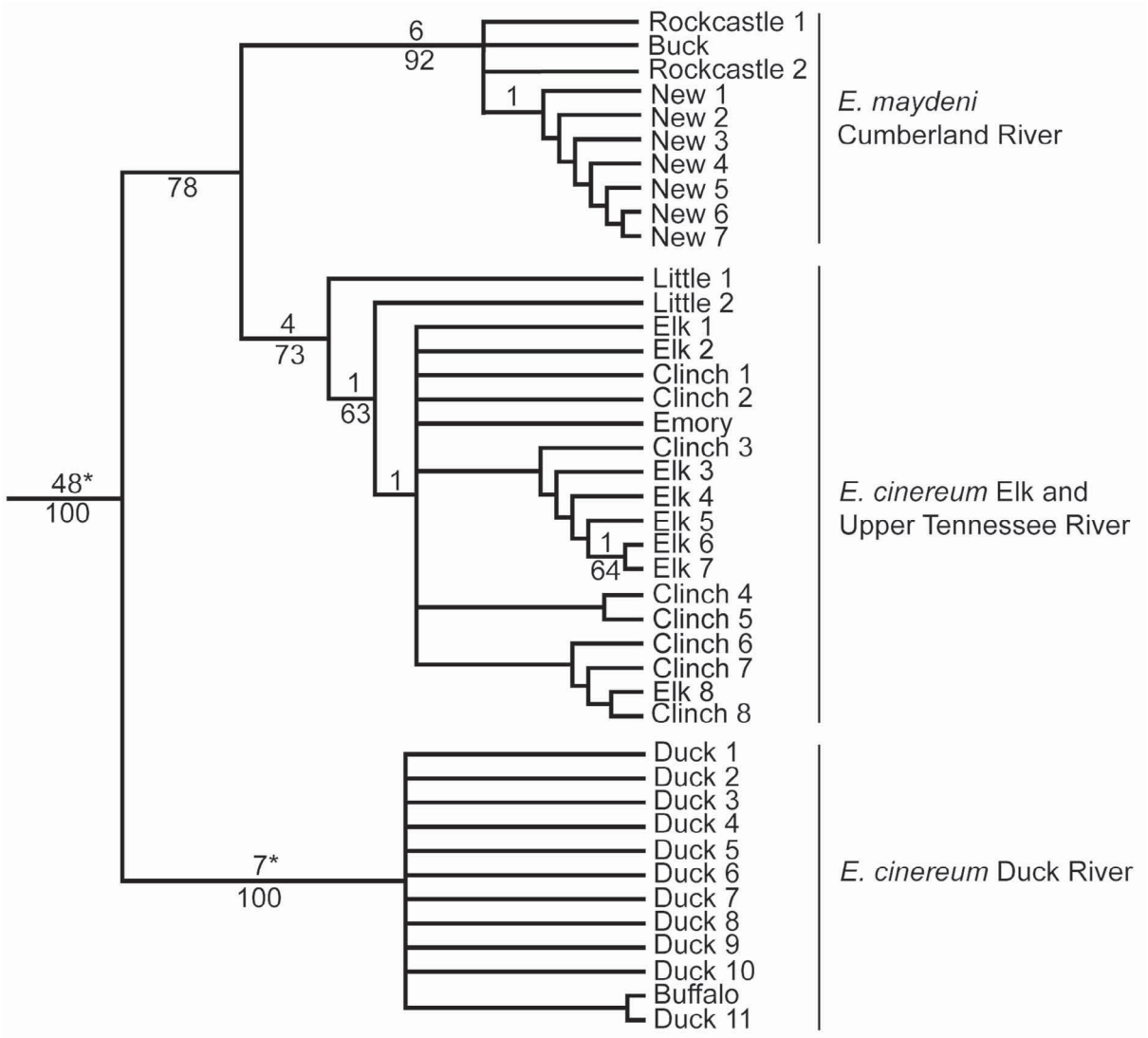

FIGURE 7. Strict consensus of *Etheostoma cinereum* complex cytochrome *b* haplotypes. Bootstrap and decay support is listed above and below each internode. An asterisk indicates nodes recovered with posterior probability values >95% in a Bayesian analysis with a similar topology.

Of the 1362 base pairs examined for RAG1, 68 were variable, 11 of which were parsimony informative. A maximum parsimony analysis identified a single most parsimonious tree with a length of 72 steps (CI = 0.96, RI = 0.91) that had an identical topology as the Bayesian analysis (Fig. 8). Monophyly of *E. cinereum* complex RAG1 sequences was supported by low bootstrap and decay support. Cumberland specimens formed a clade with moderate decay and bootstrap support and high posterior probability. This Cumberland clade was sister to all other *E. cinereum* with low bootstrap and decay support and posterior probability >95%. Specimens from the Upper Tennessee River also formed a clade with moderate decay and bootstrap support and high posterior probability. A clade containing the specimens from the Duck and Elk also had moderate bootstrap and decay support with high posterior probability. The clade containing Duck and Elk specimens showed no geographic concordance. Within clade divergence for RAG1 was 0.0%. Highest mean divergence among clades for RAG1 was 0.6% between Cumberland and Upper Tennessee specimens. All other among clade divergence was 0.4%.

Discussion

While there is overlap in ranges of meristic variables among populations, the rejection of the null hypothesis in all ANOVAs suggests there are meristic differences among populations as suggested by Shepard & Burr (1984).

Specimens from the Cumberland had more anomalous modal values for variables examined than any other population suggesting it represents the most meristically divergent population of the *E. cinereum* complex (Table 1). Shepard & Burr (1984) also identified modal differences in vertebrae among populations with Cumberland having 41, Duck having 43, and Upper Tennessee having 42.

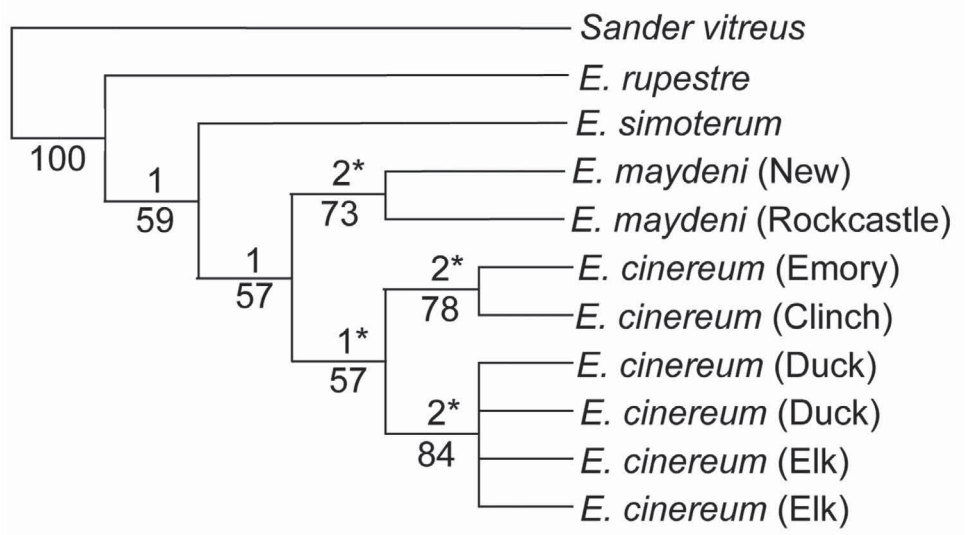

FIGURE 8. Single most parsimonious tree with a length of 72 steps (CI = 0.96, RI = 0.91) generated by analysis of Recombination Activation Gene 1 (RAG1) sequence data. Bootstrap and decay support is listed above and below each internode, respectively. An asterisk indicates nodes recovered with posterior probability values >95% in a Bayesian analysis with an identical topology.

Morphometric data for males also suggests divergence among populations with no overlap among Upper Tennessee and Elk specimens and only a low degree of overlap between Duck and Cumberland populations in scatterplots for PC 2 and PC 3 (Fig. 3). The lack of overlap between Cumberland females with those of the Elk or Upper Tennessee suggests divergence among these populations in scatterplots of PC 2 and PC 3 (Fig. 4).

Shepard & Burr (1984) identified red pigment on the lips of specimens from the Cumberland River based on a small number of observations of live, recently preserved, or live-photographed individuals and noted it as a character indicative of possible further taxonomic division with the examination of more live nuptial specimens. The examination of live specimens of multiple age classes during spawning as well as non-spawning seasons from populations across the range of the *E. cinereum* complex provides a greater foundation for describing the color differences in specimens from different populations. Presence of red pigment in the lips of all specimens examined and its conspicuous nature over most of the surface area of the lips in nuptial males from the Cumberland and only faint red on a small area of the lips of only the largest nuptial males from the Duck and Elk and complete lack of red pigment in Upper Tennessee populations (Fig. 5) represents a diagnosable morphological character for Cumberland populations suggesting this is an evolutionary species under the Morphological Species Concept (Cronquist 1978) and the diagnosable version of the Phylogenetic Species Concept (Nixon & Wheeler 1990). While it may be hypothesized that the Duck and Elk specimens represent intergrades for pigmentation of the lips, the strongly supported monophyly of the Cumberland specimens for both cyt *b* (Fig. 7) and RAG 1 (Fig. 8) along with greater among vs. within clade pairwise divergence rejects intergradation as there is no evidence of recent gene flow between Cumberland and other populations. Furthermore, these populations inhabit drainages notorious for endemic darters (see Page 1983, Etnier & Starnes 1993) suggesting their geographic isolation is not a recent phenomenon but rather has provided a long-term barrier to gene flow between populations.

Monophyly of all *E. cinereum* cyt *b* haplotypes despite the inclusion of outgroups from nearly all subgenera of darters rejects introgression as an explanation for the pattern of variation in cyt *b* found in Powers *et al.* (2004) and in this study. Separate analyses for cyt *b* and RAG 1 each identify three clades within the *E. cinereum* complex largely congruent with geographic distribution, but suggest different relationships among these clades (Fig. 6–8). The placement of the Elk River specimens also differs among the analyses with cyt *b* embedding Elk specimens within the Upper Tennessee clade while RAG1 produces a clade of Elk and Duck specimens. No decay and low to

moderate bootstrap support for the sister relationship of Cumberland and Upper Tennessee/Elk clades for cyt *b* and moderate decay and low bootstrap support for the sister relationship of Upper Tennessee and a Duck/Elk clade for RAG1 provide ambiguous hypotheses of relationships among populations. Furthermore, the ambiguous placement of Elk specimens suggests that isolation and lineage sorting of populations in the Duck, Elk, and Upper Tennessee rivers may be less complete than suggested by Powers *et al.* (2004) and leaves the evolutionary relationships of these populations unclear. Despite the ambiguous results regarding the relationships of specimens from the Duck, Elk, and Upper Tennessee rivers, DNA sequence data from specimens from the Cumberland River were recovered as monophyletic for both cyt *b* with high decay and bootstrap support and RAG1 with moderate decay and bootstrap support and high posterior probability. The monophyly of specimens from the Cumberland also suggests they represent an evolutionary species under the monophyly version of the Phylogenetic Species Concept as outlined by Rosen (1978).

The totality of evidence presented here leaves the systematic status of *E. cinereum* in the Duck, Elk, and Upper Tennessee rivers unclear. Further investigations examining more rapidly evolving markers such as microsatellites may reveal patterns of recent and historical gene flow able to clarify the evolution of these populations. However, the data presented here suggest that the extant Cumberland populations of the *E. cinereum* complex represent an evolutionary species under all versions of the Phylogenetic Species Concept, the Morphological Species Concept, and as a unique gene pool morphologically distinguishable and geographically isolated, the Biological Species Concept (Mayr 1996). Thus, we describe the Cumberland River form of the *E. cinereum* complex as a new species.

Etheostoma maydeni Powers and Kuhajda n. sp.

Redlips Darter

Holotype. USNM 403641, adult male, 79.7 mm, New River at U.S. Hwy 27 near New River, Scott County, Tennessee, (36.3827° N, 84.5529° W), 7 March 2006, S.L. Powers, B.R. Kuhajda.

Paratopotypes. UAIC 15059.02 (5, 55.9–68.7 mm) taken with holotype. UAIC 15058.02, (1, 55.0 mm), 4 March 2005, S.L. Powers, B.R. Kuhajda. INHS 33321 (2, 30.9–32.3 mm) 18 August 1994, L.M. Page, S.M. Phelps.

Paratypes. UT 91.4886 (3, 66.9–72.1 mm), Smoky Creek 3 km SW Smoky Junction, Scott County, Tennessee (36.2679° N, 84.3911° W), 10 July 1996, J.T. Baxter. SIUC 24738 (2, 54.6-77.3 mm), Big South Fork Cumberland River at mouth of Parched Corn Creek, Scott County, Tennessee, (36.5562° N, 84.6697° W), 7 September 1995, B.M. Burr. SIUC 61655 (71.3 mm), Big South Fork Cumberland River at Blue Heron, McCreary County, Kentucky, (36.6703° N, 84.5492° W), 24 August 2005, M.R. Thomas. SIUC 61628 (2, 46.3–66.7 mm), Big South Fork Cumberland River at Blue Heron, McCreary County, Kentucky, (36.6703° N, 84.5492° W), 20 November 2005, M.R. Thomas.

Diagnosis. The *Etheostoma cinereum* complex is diagnosed from all other darters by having the combination of an elongated, pointy snout, rust to faint red spots in 4 horizontal rows dorsolaterally on the side of the body, dark brown to black oval to rectangular lateral blotches expanding to faint diagonal bands on the side of the body, red pigment on interradial membranes of the soft dorsal fin, and a distal red band of pigment in the spinous dorsal fin. For further diagnoses of this complex (a.k.a. subgenus *Allohistium*), see Bailey & Gosline (1955) and Page (1981). *Etheostoma maydeni* is diagnosed from *E. cinereum* by a conspicuous red pigment on the external surface of the lips (Fig. 5). *Etheostoma maydeni* also has modally 11 dorsal spines, 12 dorsal rays, and 23 caudal peduncle scales, whereas *E. cinereum* has modally 12 dorsal spines, 13 dorsal rays, and 25 caudal peduncle scales. Shepard & Burr (1984) also reported modally 41 vertebrae in populations described herein as *E. maydeni* and with populations of *E. cinereum* having modally 42 or 43 vertebrae.

Description. Coloration and body shape of *E. maydeni* are depicted in Fig. 5A. The largest specimen examined for this study was 99.1 mm SL. Snout elongated; gill membranes separate; nape, breast and prepectoral area unscaled; belly and opercle scaled; cheek scaled but scales are often deeply imbedded and inconspicuous. Lateral-line scales 52(2), 53(4), 54(3), 55(2), 56(4), 57(5), 58(6), 59(4), 60(4), 61(3), 62(2), 63(1); dorsal spines 11(29), 12(11); dorsal rays 12(30), 13(10); anal rays 7(3), 8(27), 9(10); transverse scales 18(3), 19(4), 20(13), 21(9), 22(6), 23(5); caudal-peduncle scales 21(1), 22(7), 23(15), 24(7), 25(8), 26(2).

Coloration. External surface of lips in live nuptial males mostly brilliant red. Head largely cream to light brown in color with black to dark brown preorbital and postorbital bar, dark dorsal dark brown pigment arranged in vermiculated pattern, ventral half of head generally lacking dark pattern, but often with faint blue cast over gill membranes that can become dark blue to black in peak males. A series of 10–13 dark brown to black oval to rectangular lateral blotches expanding to faint diagonal bands on the side of the body. Tan to light brown ventral to the lateral blotches. Rust to faint red spots in 4 horizontal rows dorsolaterally on the side of the body surrounded by tan to light brown with 7–10 faint dorsal saddles. Pelvic and anal fins ranging from clear in non-nuptial males to bright blue to dark blue or black in nuptial males. Caudal fin largely clear with bright blue cast near dorsal and ventral origins and red spots on interradial membranes. Interradial membranes of soft-dorsal fin with bright red pigment forming a contiguous band sometimes degrading to a series of bright red spots distally. Spinous dorsal fin with a bright red distal margin and interradial bands or vermiculation transitioning from black or brown proximally to red distally.

Ecology. The ecology of *E. maydeni* was described by Shepard & Burr (1984) along with the rest of the *E. cinereum* complex. Cumberland River system populations (i.e., *E. maydeni*) were noted as having slower growth than other populations, males outnumbering females rather than the opposite for other populations, and a greater reliance on burrowing mayflies (Ephemeroptera: *Ephemera*) and oligochaetes in their diet with a lower proportion of Chironomidae compared to other populations. Specimens for this study were collected largely in water approximately 0.5–1 m deep over large rocks and course woody debris with slow current adjacent to faster current.

Distribution. *Etheostoma maydeni* is restricted to large tributaries of the Cumberland River below Cumberland Falls. The mainstem and large tributaries of the Big South Fork and Rockcastle rivers are home to the largest populations. The Buck Creek population was considered extirpated by Shepard & Burr (1984), but several specimens have been collected in recent years, suggesting the species has made a comeback in the stream. The Red and Stones river populations of the *E. cinereum* complex were considered extirpated by Shepard and Burr (1984) and despite sampling in these drainages for this and other projects by the authors and many other researchers, we have no evidence to contradict this assertion. The status of populations in the Obey and Roaring rivers was considered unknown by Shepard & Burr (1984) due to absence of the species from collections in these drainages since the early 1970s. Sampling in these rivers for this and other studies by the authors and other researchers has not yielded specimens, suggesting these populations are extirpated. However, given the recent rediscovery of *E. cinereum* in the Elk River after a 30-year absence, it is possible that these populations may persist in low numbers in what appears to be suitable habitat for the species.

Etymology. The name *maydeni* is in honor of Dr. Richard L. Mayden, a prominent ichthyologist, mentor and friend of the authors. His studies of North American fishes include ecology, biogeography, conservation, and systematics. The common name "Redlips Darter" refers to the conspicuous red pigment on the surface of the flesh surrounding the premaxilla and mandible often referred to as lips.

Comparisons. Pigmentation differences between *E. maydeni* and *E. cinereum* appear to be restricted to bright red pigment on the lips for the former. *Etheostoma maydeni* has modally fewer dorsal spines, dorsal rays, and caudal-peduncle scales than *E. cinereum* (Table 1).

Comments. Photos of *E. maydeni* have been published as *E. cinereum* (Kuehne & Barbour 1983; Shepard & Burr 1984).

Materials Examined

Morphological analyses

Etheostoma maydeni—USNM 403641, 1; UAIC 15059.02, 8; UAIC 12458.03, 2; UAIC 14930.01, 8; UAIC 15057.01, 1; UT 91.5028, 1; UT 91.4881, 3; UT 91.4886, 3; UT 91.4859, 1; RC REJ555, 2; RC REJ557, 1; SIUC 82115, 2; SIUC 81803, 1; SIUC 81954, 2; SIUC 82301, 1; SIUC 82068, 1; SIUC 82090, 1.

Etheostoma cinereum—Duck R. system—JFBM 37545, 1; NLU 29692, 5; NLU 39445, 6; UAIC 15056.01, 2; RC SLP 0903, 4; RC SLP 0917, 9; UAIC 10463.09, 2; UAIC 11314.15, 1; UAIC 13143.24, 2; UAIC 15763.01, 5; UAIC 12771.19, 3; UT 91.736, 1. Elk R. system—UAIC 15065.01, 2; RC SLP0918, 9. Upper Tennessee R. system—RC NMB535, 2; RC NMB835, 3; RC DAN08115, 1; RC DAN08116, 2; RC SLP0919, 3; UAIC 5818.16, 2; UAIC 8878.01, 2; UAIC 15062.01, 2; UT 91.593, 7.

Molecular analyses

Cyt *b*. *Etheostoma maydeni*—UAIC 12354.12, AY560350; AF045349 from Song et al. (1998); UAIC 13476.10, AY560349; UAIC 13734.01, AY560348, AY560351; UAIC 15058.02, JN837014; RC SLP0506, JN837011, JN837013; RC SLP0507, JN837012;RC MRT1143, JN837010. *Etheostoma cinereum*—R. system—UAIC 12771.19, AY560356–7; UAIC 13679.02, AY560352–4; UAIC 13662.01, AY560355; RC SLP0916, JN837015–9; RC SLP0903, JN837020: Elk R. system—RC SLP0918, JN837021–8: Upper Tennessee R. system—UAIC 8591.16, AY560359–60; UAIC 12433.09, AY560358; RC DAN08115, JN837036; RC DAN08116, JN837029, JN837031; RC SLP0919, JN837032–3, JN837035; UAIC 15062.01, JN837030, JN837034.

RAG1. *Etheostoma maydeni*—UAIC 12354.12, JN837040; UAIC 15058.02, JN837039. *Etheostoma cinereum*—R. system—RC SLP0903, JN837037; RC SLP0916, JN837038: Elk R. system—RC SLP0918, JN837043–4: Upper Tennessee R. system—RC DAN08115, JN837041; RC SLP0919, JN837042.

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